

An In Vivo and In Vitro Model of *Plasmodium falciparum* Rosetting and Autoagglutination Mediated by *varO*, a Group A *var* Gene Encoding a Frequent Serotype[▽]

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Received 22 July 2008/Returned for modification 7 September 2008/Accepted 11 September 2008

In the *Saimiri sciureus* monkey, erythrocytes infected with the *varO* antigenic variant of the *Plasmodium falciparum* Palo Alto 89F5 clone bind uninfected red blood cells (rosetting), form autoagglutinates, and have a high multiplication rate, three phenotypic characteristics that are associated with severe malaria in human patients. We report here that *varO* parasites express a *var* gene having the characteristics of group A *var* genes, and we show that the *varO* Duffy binding-like 1 α_1 (DBL1 α_1) domain is implicated in the rosetting of both *S. sciureus* and human erythrocytes. The soluble *varO* N-terminal sequence (NTS)-DBL1 α_1 recombinant domain, produced in a baculovirus-insect cell system, induced high titers of antibodies that reacted with *varO*-infected red blood cells and disrupted *varO* rosettes. *varO* parasites were culture adapted in vitro using human erythrocytes. They formed rosettes and autoagglutinates, and they had the same surface serotype and expressed the same *varO* gene as the monkey-propagated parasites. To develop an in vitro model with highly homogeneous *varO* parasites, rosette purification was combined with positive selection by panning with a *varO* NTS-DBL1 α_1 -specific mouse monoclonal antibody. The single-variant, clonal parasites were used to analyze seroprevalence for *varO* at the village level in a setting where malaria is holoendemic (Dielmo, Senegal). We found 93.6% (95% confidence interval, 89.7 to 96.4%) seroprevalence for *varO* surface-reacting antibodies and 86.7% (95% confidence interval, 82.8 to 91.6%) seroprevalence for the recombinant NTS-DBL1 α_1 domain, and virtually all permanent residents had seroconverted by the age of 5 years. These data imply that the *varO* model is a relevant in vivo and in vitro model for rosetting and autoagglutination that can be used for rational development of vaccine candidates and therapeutic strategies aimed at preventing malaria pathology.

Plasmodium falciparum malaria is a major public health burden in intertropical areas, with up to 600 million cases and more than 2 million deaths each year, mainly African children (8). A pathological hallmark of *P. falciparum* infections is sequestration of mature intraerythrocytic parasite stages in the microvasculature of vital organs. Sequestration results from cytoadherence of *P. falciparum*-infected red blood cells (iRBC) to microvascular endothelial cells or to circulating blood cells. Rosetting (i.e., the capacity of iRBC to bind uninfected red blood cells [RBC]) has been consistently associated with severe malaria in African children (12, 23, 30, 59, 67, 85). Platelet-mediated clumping of *P. falciparum* iRBC has been associated with severe malaria in many studies (20, 60, 64, 88) but not in all studies (2, 3). Importantly, children with severe malaria do not have rosette-disrupting antibodies (12). The mechanism by which rosetting contributes to the severity of infection may

result from occlusion of the microvasculature (36, 54) and/or from a particularly high parasite multiplication rate, which may be favored by efficient invasion of the uninfected RBC in the rosettes by bursting merozoites (47).

Analysis of the molecular basis of cytoadherence has highlighted the key role played by the variant *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) encoded by the *var* multigene family (for a review, see reference 39). PfEMP1 adhesins are high-molecular-mass proteins with a large extracellular region consisting of Duffy binding-like (DBL), constant (C2), and cysteine-rich interdomain region (CIDR) modules. Specific sequence signatures permit grouping of DBL domains into seven distinct classes (DBL α , DBL α_1 , DBL β , DBL γ , DBL δ , DBL ϵ , and DBL χ) and CIDR domains into four classes (CIDR α , CIDR α_1 , CIDR β , and CIDR γ) (39, 40, 65, 78). Based on 5' and 3' noncoding sequences, domain combinations, chromosomal location, and gene orientation, *var* genes were classified into three major groups, groups A, B, and C, and two intermediate groups, groups B/A and B/C (39, 40, 65, 78).

Based on the limited number of genes associated thus far with rosetting, it appears that this phenomenon is mediated by

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[▽] Published ahead of print on 22 September 2008.

a small subset of PfEMP1 variants (9), each of which is involved in a specific interaction(s) with host molecules (for reviews, see references 27 and 50), including RBC surface receptors (29, 70, 86) and serum components (21, 26, 30, 49, 50, 71, 79). To date, two in vitro rosette-forming variants have been studied in detail. The first variant, designated R29, expresses a group A *var* gene that codes for a PfEMP1 adhesin that binds to complement receptor 1 (CR1)/CD35 (68). The second variant, designated FCR3S1.2, forms giant rosettes and expresses a PfEMP1 molecule that binds to diverse host receptors, including heparan sulfate, blood group A, immunoglobulin M (IgM), PECAM-1/CD31, and CD36 (14, 15, 75). In contrast to that of R29, the FCR3S1.2 *var* gene does not belong to group A (38). Expression of individual modules from both variants has shown that the N-terminal DBL1 α domain of each variant mediates rosetting (15, 68). R29 and FCR3S1.2 are antigenic variants of the FCR3/IT4 line, which is poorly infectious for nonhuman primates (25), which hampers in vivo experimental studies with rosette-forming parasites.

We developed an in vivo experimental model of rosetting in the *Saimiri sciureus* monkey using the varO antigenic variant of the Palo Alto 89F5 clone, which forms rosettes and autoagglutinates that circulate in the peripheral blood of splenectomized animals (22, 24, 46). This allowed us to show that varO parasites have a higher in vivo multiplication rate than the isogenic, nonrosetting, antigenic variant varR parasites (47). We report here that the *varO* gene expressed by 89F5 varO parasites has the main features of the genes belonging to the group A/UpsA subset, which is frequently associated with severe malaria in African children (35, 41). Due to high costs associated with *S. sciureus* studies and the paucity of specific reagents available for this animal species, we developed an in vitro varO model using human RBC culture. We show here that human RBC culture-adapted 89F5 varO parasites express the same *varO* gene, have the same rosetting and autoagglutination phenotypes, and have the same surface serotype as *Saimiri*-propagated parasites. To establish a continuous in vitro culture of parasites uniformly expressing the *varO* gene, rosette enrichment was combined with positive selection by panning with a mouse monoclonal antibody (MAb) raised to a soluble varO N-terminal sequence (NTS)-DBL1 α_1 recombinant domain. The single-variant preparations obtained were used to investigate the seroprevalence of antibodies against varO parasites in Dielmo, a Senegalese village located in a region where malaria is holoendemic. Very high levels of seroprevalence were observed for varO-infected RBC (varO-iRBC) and for the recombinant rosetting domain. The varO model thus is a pertinent rosetting-autoagglutination model and, indeed, the only system for which both a nonhuman primate host and an in vitro model are available to guide the design of rational intervention strategies and vaccine candidates.

MATERIALS AND METHODS

Parasites. The 89F5 parasite clone was obtained by cloning by limiting dilution (in human RBC cultures) the uncloned varO antigenic variant of the *Saimiri*-adapted Palo Alto strain of *P. falciparum* (also designated FUP/SP) (24, 47). The 89F5 clone was then inoculated into a naive splenectomized animal and further propagated in splenectomized *Saimiri* monkeys by serial blood passage (44). RBC infected with the uncloned varO line or with the 89F5 varO clone form rosettes and spontaneously autoagglutinate in the absence of immune serum (24, 46). The nonrosetting varR clone is an isogenic antigenic variant of the Palo Alto

FUP/SP line obtained using a varO-infected monkey under immune pressure (24, 45, 46).

***Saimiri* monkey infection and blood sampling.** Male adult *Saimiri* monkeys were used in this study. The procedures used for housing, animal handling, and blood sampling have been described previously (24, 45–47). Nonimmune and immune (anti-varO and anti-varR) *Saimiri* sera were obtained as described previously (47). For RNA and DNA preparation, peripheral blood was collected by venipuncture from anesthetized animals when parasitemia was $\geq 20\%$.

Cloning and sequencing of the *var* gene expressed by 89F5 varO parasites. In order to obtain RNA preparations devoid of the nonspecific abortive *var* transcripts present in early stages (74) and with host RNA depleted, infected blood samples collected from infected *Saimiri* monkeys were treated as follows. After the white blood cells were removed with a CF11 column (31), the mature stages present in the blood samples were lysed with 5% sorbitol, while the young stages were allowed to mature in complete culture medium for 24 h before they were harvested by centrifugation. RNA was extracted from the mature parasite stages as described previously (19). DNase I-treated RNA preparations were used for reverse transcriptase PCRs (RT-PCRs). Comparative RT-PCRs with varO and varR parasites were used to ascertain the variant-specific expression profile. The first *varO*-specific fragment cloned was derived from the DBL3 γ domain, which was cloned from RT-PCR-generated fragments using the UNIEBP5' and UNIEBP3' primers described elsewhere (45, 62). This generated the nucleotide sequence from position 3799 to position 4266 of the *varO* gene. The *varO* DBL1 α fragment was then amplified by RT-PCR using the universal α -AF and α -BR primers (81) and cloned using a TOPO TA cloning kit (Invitrogen). The DBL1 α fragment included nucleotide positions 577 to 959 of the *varO* sequence. Specific primers based on the DBL1 α and DBL3 γ fragments were then used to clone and sequence the entire *varO* gene using (i) chromosome walking, which produced flanking fragments for both domains, and (ii) RT-PCR performed with specific and conserved primers, which generated DBL1-DBL3 and DBL3-exon II fragments. Chromosome walking was performed with a Universal genome walker kit (Clontech) using *varO* DBL1 α - or *varO* DBL3 γ -specific primers (Table 1). RT-PCR was used to amplify a 3,071-bp fragment from *varO* RNA with the DBL1-specific primer DBL1O-fo851 and the *varO* DBL3-specific primer DBL3O-rev3894 and to amplify a 3,249-bp fragment with the *varO* DBL3-specific primer DBL3O-fo4129 and exon II conserved primer L5 (14). The PCR fragments were directly sequenced to avoid possible cloning artifacts in the sequence. The cDNA sequence confirmed the sequence data obtained by chromosome walking. The available *varO* cDNA sequence codes for an 7,378-bp open reading frame starting with the initiator codon ATG. The exon II sequence is a partial sequence.

PfEMP1varO sequence analysis and homology search with the databases. PfEMP1varO protein domain sequence boundaries were defined by using previously described criteria (65, 78, 80). Type-specific consensus motifs were used to classify individual varO domains. For each domain, sequence homology searches using *P. falciparum* isolate 3D7, IT4/25/5, HB3, Dd2, and Ghana and chimpanzee malaria parasite *Plasmodium reichenowi* partial genome and protein sequence databases were performed using the BLAST tools available and specialized *Plasmodium* databases. Sequences with high levels of similarity were extracted. Translated sequences were aligned using the ClustalW program (DNASTAR Lasergene software, V7.1.0). The cysteine/position of limited variation (PoLV) classification was used to assign the varO DBL1 α domain (9, 10).

In vitro culture of 89F5 varO parasites in human RBC. Cryopreserved blood of *Saimiri* monkeys infected with 89F5 varO parasites was thawed and used to establish an in vitro culture with human RBC. The parasites were cultivated and maintained in continuous culture in human O⁺ RBC (blood bank of Etablissement Français du Sang [EFS], Rungis, France), adjusted to 5% hematocrit (82), in RPMI 1640 medium (Invitrogen) supplemented with 10% human AB⁺ serum using an atmosphere containing 5% O₂, 5% CO₂, and 90% N₂. Routine screening of cultures for *Mycoplasma* contamination by PCR using a VenorGeM mycoplasma detection kit (Biovalley) was negative. Rosetting parasites were enriched once a week by centrifugation (30 s, 1,000 \times g) on ice-cold Ficoll (1.077 g/ml; Lymphoprep; Abcys) as previously described (48). Rosette formation was monitored after iRBC nuclei were stained with Hoechst dye (Hoechst 33342; Molecular Probes). The rosetting rate was calculated by determining the percentage of rosette-forming iRBC present in the mature parasite population. A weekly enrichment procedure kept the rosetting rate above 90%.

Selection of varO-iRBC by panning with a varO-specific mouse MAb. To select for the varO phenotype in a heterogeneous population of rosette-forming parasites, varO-iRBC were positively selected by panning with D15-50, a mouse MAb raised to the rNTS-DBL1 α_1 domain of varO (M. Guilloitte, I. Vigan-Womas, A. Juillerat, S. Igonet, F. Marchand, F. Nato, G. Bentley, and O. Mercereau-Pujalon, unpublished data). Briefly, rosette-forming iRBC were first treated with 10 μ g/ml dextran sulfate (molecular weight, >500,000; Sigma) to

TABLE 1. Primer sequences used for *varO* gene sequencing and for detection of *varO* expression in 89F5 varO parasite cultures

Primer ^a	Sequence (5'–3')	Primer positions (bp)
Primary PCR		
DBL1O-rev639	CTTGTAAGTCCTTTTGTACTTTATCATCC	639–667
DBL1O-rev853	TCCGTGACTGCTAAAATGTAGAGTACC	853–879
DBL1O-fo851	ATGGTACTCTACATTTTAGCAGTCACGG	851–878
DBL3O-rev3894	ATATTTATACCAAGCAAAATGTGTTTCTATA	3894–3924
DBL3O-rev4095	GAAGAAAGAAAGTAAGGAAAGAAAAATCC	4095–4124
Nested PCR		
DBL1O-rev624	GTAATTTATCATCCTTATTAGGTAAAAAC	624–652
DBL1O-rev844	CTGCTAAAATGTAGAGTACCATCTGAACC	844–872
DBL1O-fo864	TTTTAGCAGTCACGGAAGTGC GGCC	864–889
DBL3O-rev3846	CTCTGTTCTTATATCTTCTTTTCTTTTATA	3846–3876
DBL3O-fo4129	GAATGGTGAATGAACATGGAAAGGAG	4129–4155
L5 (exonII)	CCATCTTCATATTCATTTCTGA	
<i>varO</i> gene expression		
5ID3Bfo	GCCCAATTGGATCCACAAAACAAAGCATATAAAG	5764–5797
5DBL5Bfo	TGTAAGAAAGTAGGATCCAGGTAGTTGTCCAGAA	6085–6117
VarO3exon2Brev	ATATCTATTTGATGATTTCCGGGGTAGG	7216–7242

^a rev, reverse primer; fo, forward primer.

dissociate rosettes. iRBC were then magnetically selected on a MACS column (Miltenyi BioTec) (61), eluted, resuspended in phosphate-buffered saline (PBS) with 2% human AB⁺ serum, and placed in a culture flask (75 cm²; Corning) that had been coated overnight at 4°C with MAb D15-50 (10 ml of a 100-μg/ml solution in PBS) and saturated for 2 h at room temperature with PBS containing 2% bovine serum albumin. Binding was performed at 37°C for 2 h with gentle rocking every 30 min. Unbound iRBC were removed by washing the preparation with the same medium, and binding of varO-iRBC was checked using an inverted microscope. After addition of fresh RBC along with complete culture medium, the parasites were allowed to invade overnight. The culture was then transferred to new flasks and incubated under standard conditions. The percentage of varO-positive iRBC in the culture was routinely assessed by performing surface immunofluorescence assays (S-IFA) with anti-rNTS-DBL1α₁ mouse serum or MAb D15-50.

***varO* gene expression in cultured 89F5 varO parasites.** RT-PCR was performed using total RNA extracted with the Trizol reagent (Invitrogen) from synchronized 89F5 varO parasite cultures at the trophozoite and early-schizont stages. The 5DBL5Bfo-VarO3exon2Brev and 5ID4Bfo-VarO3exon2Brev specific primer pairs (Table 1) were used. One-half of the RNA preparations were treated with RNase-free DNase I (Invitrogen) before RT-PCR amplification. RT-PCRs were performed using the Access RT-PCR introductory system (Promega) according to the manufacturer's instructions. PCR products were analyzed by agarose gel electrophoresis.

Plasmid constructs and production of recombinant baculovirus. To overcome expression problems caused by the codon usage bias of *P. falciparum* genes, codon-optimized versions of domain-encoding sequences were synthesized. Recoded DBL1α₁ optimized for *Homo sapiens* codon usage (positions 96 to 398 of the deduced varO protein sequence), designated rhDBL1α₁, has been described elsewhere (72). Insect cell-optimized coding sequences of the varO CIDRγ, DBL2βC2, and DBL5β domains (positions 399 to 835, 821 to 1241, and 2031 to 2264 of the deduced varO protein sequence, respectively) were custom-made (Biometech, Evry, France). Insect cell-optimized NTS-DBL1α₁ (positions 1 to 487 of the deduced varO protein sequence) was synthesized by GeneCust (Evry, France). For each domain, all potential N-glycosylation sites were mutated (NXS/T to NXA), as were restriction sites that could hinder cloning manipulations. The constructs also contained a coding sequence for a six-histidine tag 3', upstream from, and in frame with a stop codon. The synthesized recombinant genes, designated rhDBL1α₁, rNTS-DBL1α₁, rCIDRγ, rDBL2βC2, and rDBL5β, were cloned in the pMelBacA plasmid (Invitrogen) downstream from and in phase with the honeybee mellitin signal sequence. Each sequence was confirmed base by base for both strands.

Spodoptera frugiperda (Sf9; Invitrogen) and *Trichoplusia ni* (High-5; Invitrogen) insect cells were grown in monolayer or suspension cultures at 27°C, in SF-900-II serum-free medium (Gibco-BRL) supplemented with 4 mM glutamine (Gibco-BRL), 5% fetal bovine serum, and 50 μg/ml gentamicin. For each do-

main, recombinant baculovirus was generated using the Bac-N-Blue transfection and expression system (Invitrogen) according to the manufacturer's protocol. Viral stocks were produced in Sf9 insect cells and stored at 4°C. The recombinant viral clones were confirmed by PCR and sequencing.

Cell surface expression of varO recombinant domains and erythrocyte binding assay. Briefly, 5 × 10⁶ High-5 cells grown in a monolayer were infected with recombinant baculovirus. After 90 min of incubation at 27°C, the infection medium was removed, and infected cells were cultured in complete culture medium for 3 days. Infected cells expressing rhDBL1α₁, rCIDRγ, rDBL2βC2, or rDBL5β were harvested and analyzed in order to determine surface expression of the recombinant domain by S-IFA using a mouse anti-His tag MAb (Novagen), followed by Alexa Fluor 488-conjugated goat anti-mouse IgG(ab')₂ (Molecular Probes). Staining was analyzed by fluorescence microscopy (Leica DM4500B) or by flow cytometry (FACS LSR1; Becton Dickinson). Erythrocyte binding on insect cells expressing varO recombinant domains was assessed using 1 × 10⁶ infected High-5 cells washed twice with RPMI 1640 and incubated with human O⁺ or A⁺ RBC in RPMI 1640 supplemented with 10% human serum for 3 to 5 h at 27°C. An aliquot of a cell suspension was mounted on a glass slide, and rosette formation was monitored by microscopy.

Production of soluble recombinant domains and mouse immunization. To produce a soluble rNTS-DBL1α₁ domain, suspension cultures of Sf9 cells were infected with recombinant baculovirus harboring the rNTS-DBL1α₁ construct. After 3 days of incubation, the soluble secreted protein was harvested from culture supernatants and purified as described previously (7). The sequence of the recombinant protein was verified by mass spectroscopy, and its purity was assessed by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and immunoblotting. The N-terminal sequence of the recombinant protein was determined and was shown to match the predicted sequence after cleavage of the mellitin signal sequence. The DBL3γ732 recombinant protein was obtained as described previously (17).

Outbred (OF1) female mice (6 to 8 weeks old; Charles River, France) were immunized by subcutaneous injection at 3-week intervals of 10 μg of soluble rNTS-DBL1α₁ or DBL3γ732 protein in the presence of Freund's adjuvant (complete Freund's adjuvant for the first immunization and incomplete Freund's adjuvant for the booster immunizations). Sera were collected 10 days after the third injection, pooled, and analyzed by enzyme-linked immunosorbent assay (ELISA), immunoblotting, S-IFA, and rosette dissociation assay. Mouse MAb D15-50 was obtained from a mouse immunized with rNTS-DBL1α₁ as described elsewhere (55).

Collection of human serum from malaria-exposed and non-malaria-exposed individuals. A longitudinal study was carried out in Dielmo (Senegal), a village located in an area where malaria is holoendemic (the study design has been described previously [58, 66, 84]). For this substudy, we used serum samples collected from 235 (ages, 1 to 85 years; mean age, 23 years) of the 247 villagers living in Dielmo from July to September 1992. During the 1992 rainy season, the

entomological inoculation rate was 26 ± 17 infective bites/person/month. Serum samples were stored at -20°C until they were used at the Institut Pasteur in Paris, France. This longitudinal study is part of a malaria immunity program and has been approved by National Council on Health Research of Senegal (reference no. 1971 MSPM/DS/DER; August 2006); it is being conducted in accordance with the Declaration of Helsinki. Informed written consent is obtained when an individual is included in the study and is orally confirmed each year by the volunteers studied. For the varO substudy, the protocol was approved by the institutional review boards of the Institut Pasteur of Paris (reference no. RBm/2006.032; September 2007) and the National Council on Health Research of Senegal (reference no. 05 MSP/DS/CNRS; February 2008). We also used non-immune human plasma obtained from healthy non-malaria-exposed adults (blood bank of EFS, Rungis, France).

S-IFA. After rosette enrichment, an aliquot of 89F5 varO rosetting parasites (rosetting rate, $>85\%$) was incubated for 30 min at 37°C with serial dilutions of mouse sera or with human plasma or *Saimiri* sera (1:20 dilution). After two washes with PBS with 2% fetal calf serum, the binding of IgG or IgM was detected with the appropriate secondary antibody diluted 1:1,000 in PBS with 2% fetal calf serum. Surface-reactive human or *Saimiri* antibodies were detected with Alexa Fluor 488-conjugated goat anti-human IgG or IgM (Molecular Probes). For mice, Alexa Fluor 488-conjugated goat anti-mouse IgG F(ab')₂ (Molecular Probes) was used. Each secondary antibody was mixed with Hoechst dye (1:1,000 dilution; Hoechst 33342; Molecular Probes) for iRBC nucleus staining. After 30 min of incubation at 37°C and two washes, parasites were fixed with 0.37% formaldehyde (Sigma), and immunofluorescence was analyzed by using fluorescence microscopy or flow cytometry. Pools of sera from nonimmune mice, humans not exposed to malaria, or naïve *Saimiri* monkeys were used as negative controls (NC).

For the seroprevalence study, a pool of 30 adult sera from Dielmo was used as a positive control (PC). Five thousand events were recorded for Hoechst dyed iRBC, and the data were processed with the CellQuest software (Becton Dickinson). For each sample, the percentage of Alexa Fluor-positive iRBC (%iRBC⁺) and the mean fluorescence intensity (MFI) were calculated. The IgG surface reactivity was expressed in arbitrary units (AU), determined as follows: $[(\%iRBC^+_{\text{sample}} - \%iRBC^+_{\text{NC}})/(\%iRBC^+_{\text{PC}} - \%iRBC^+_{\text{NC}})] \times 100$. A reactivity index that took into account the MFI was calculated as follows: $[(MFI_{\text{sample}} \times \%iRBC^+_{\text{sample}})/(MFI_{\text{PC}} \times \%iRBC^+_{\text{PC}})] \times 100$. The mean surface reactivity plus 3 standard deviations observed with plasma from 20 European donors not exposed to malaria (EFS, Rungis, France) was used as a negative cutoff value. A cutoff value of 20 AU was obtained, and individuals having surface-reactive antibody values above this value were considered positive.

ELISA using varO rNTS-DBL1 α_1 . ELISA plates (Maxisorp 439454; Nunc, France) were coated with 0.2 μg (in 100 μl) of rNTS-DBL1 α_1 protein or nonfat milk protein in PBS. After overnight incubation at 4°C , the plates were blocked with 200 μl PBS-5% nonfat milk for 1 h at 37°C . Human or *Saimiri* sera (1:100 dilution) or anti-rNTS-DBL1 α_1 mouse sera (twofold serial dilutions) were diluted in PBS-2.5% nonfat milk-0.05% Tween 20 and incubated for 1 h at 37°C . Immunoglobulin binding was detected using horseradish peroxidase-conjugated goat anti-human IgG F(ab')₂ (Cappel, France), anti-mouse IgG (Promega, France), and (for *Saimiri* sera) anti-human IgG (Promega, France). Enzymatic reactions were developed for 10 min at room temperature after addition of the trimethylbenzene-H₂O₂ substrate (KPL). Optical densities (OD) at 450 and 655 nm were determined with a multiscan plate reader (Bio-Rad). Each serum was tested in duplicate.

To study the seroprevalence of varO rNTS-DBL1 α_1 , antibody responses were expressed in AU to account for day-to-day variation and were calculated as follows: $[(OD_{\text{sample}} - OD_{\text{background}})/(OD_{\text{PC}} - OD_{\text{background}})] \times 100$. A pool of plasma from adults from Dielmo reacting strongly with the surface of varO-iRBC was used as a positive control. The mean OD plus 2 standard deviations obtained with plasma from 20 healthy European donors (EFS, Rungis, France) who had not been exposed to malaria was used as a negative cutoff value. A cutoff value of 7.76 AU was obtained for rNTS-DBL1 α_1 .

Rosette dissociation assay. An aliquot of rosette-enriched 89F5 varO parasites (20 μl in complete RPMI culture medium; rosetting rate, $>85\%$; 5% parasitemia) was incubated for 30 min at 37°C with twofold serial dilutions of mouse serum pools (range, 1/5 to 1/1,000). The rosetting rate was evaluated by light microscopy. Each serum was tested in duplicate.

Scanning electron microscopy. varO rosetting parasites were enriched using ice-cold Ficoll. A pellet containing rosetting parasites was washed in PBS, fixed for at least 1 h with 2.5% glutaraldehyde in PBS and 0.1 M cacodylate buffer, washed, and postfixed in 1% osmium tetroxide. After dehydration using a graded ethanol series, samples were critical point dried (Balzers Union CPD30) and

coated with gold-palladium (Gatan 681 ion beam coater). Samples were visualized with a JEOL JSM 6700F scanning electron microscope.

Statistical analysis. Results obtained for surface-reactive and rNTS-DBL1 α_1 -reactive antibodies were log transformed to obtain a normal distribution, and Student's *t* test was used to compare the groups studied. When the log-transformed data were not normally distributed, Wilcoxon's nonparametric test was used. Comparisons of varO surface-reactive antibodies and rNTS-DBL1 α_1 -reactive antibodies were performed by using the Spearman rank correlation test. Data were analyzed with STATA software (version 9; STATA Corporation, College Station, TX), and a *P* value of <0.05 was considered statistically significant.

Nucleotide sequence accession number. The sequence reported in this paper has been deposited in the GenBank database under Palo_Alto_varO GenBank accession number EU9082205.

RESULTS

Characterization of the varO gene expressed by mature varO parasites. To isolate the *var* gene specifically expressed by varO parasites in *S. sciureus*, RT-PCR was carried out with mRNA extracted from mature trophozoites using the degenerate α -AF and α -BR primers. Of the 49 cDNA clones obtained, 28 (57%) had the same DBL1 α sequence. This consensus sequence was completely absent from 20 and 23 cDNA clones generated from two sibling variants of the Palo Alto line (varR and varS+, respectively) (22, 24) and hence was considered specific for the *varO* trophozoite mRNA preparation. RT-PCR using the UNIEBP primers amplified a 450-bp product derived from a DBL3 γ domain that was also enriched in *varO* parasite RNA preparations. We concluded that the two predominant sequences were varO specific and originated from the major *var* gene expressed by the varO parasites; accordingly, we designated this gene *varO*. Specific primers derived from these two domains were used to amplify the entire *varO* exon I sequence and part of *varO* exon II by combining RT-PCR and chromosome walking (Table 1; also see Materials and Methods). They were also used to confirm expression specificity in independent RNA preparations from four varO- and four varR-infected animals (data not shown). Furthermore, we showed that the same *varO* gene was expressed in the uncloned Palo Alto FUP/SP varO line and in the 89F5 parasite clone derived from this uncloned population.

Analysis of the varO cDNA sequence obtained yielded an assembled 7,378-bp open reading frame (Palo_Alto_varO GenBank accession no. EU9082205). Domain identification using protein signatures defined previously (78) showed that PfEMP1varO exon I codes for five DBL domains (one DBL1 α_1 domain, three DBL β domains, and one DBL γ domain), one CIDR γ domain, one C2 domain, and three unclassified interdomain regions. The PfEMP1varO modular structure organization and amino acid sequence are shown in Fig. 1. *varO* has a typical group A (also called UpsA) signature, in particular, multiple domains and an NTS-DBL1 α_1 -CIDR γ head structure that is found only in group A *var* genes (28). Moreover, the PoLV sequence signatures of the *varO* DBL1 α_1 domain as defined by Bull et al. (9, 10) place *varO* DBL1 α_1 in group 1 (2Cys) (Fig. 2), which is found exclusively in group A *var* genes.

varO orthologs. Nucleotide alignment and deduced amino acid sequence alignment did not identify any *var* gene with a similar domain arrangement or a closely related sequence throughout the entire cDNA *varO* sequence in any complete

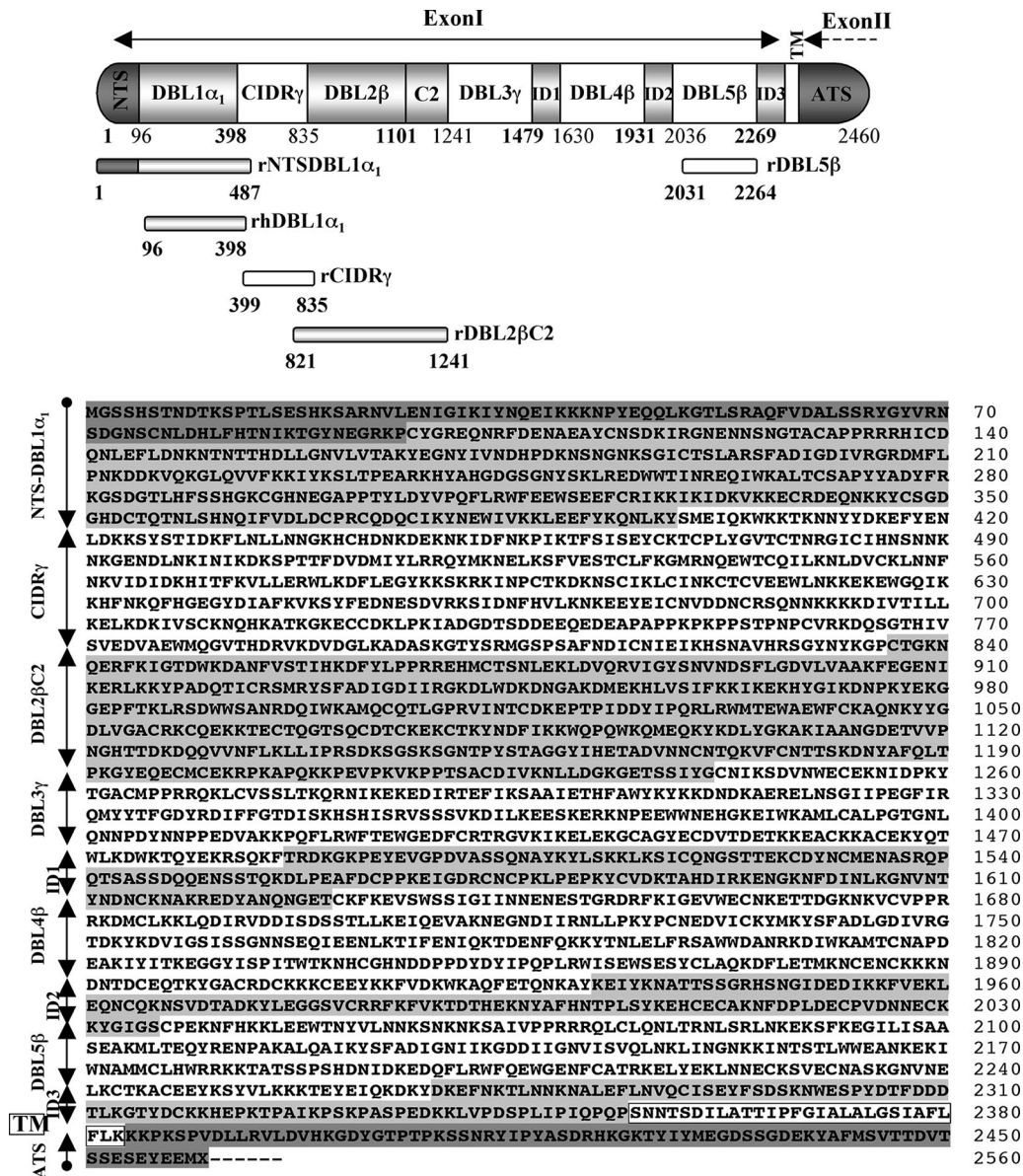


FIG. 1. Schematic diagram of the domain organization and deduced amino acid sequence for the *varO* gene expressed by Palo Alto *varO* parasites. The *varO* gene encoding the PfEMP1 *varO* adhesin was cloned and sequenced (see Materials and Methods). The *varO* cDNA sequence codes for a 7,378-bp open reading frame (2,460 amino acids) (Palo Alto *varO* GenBank accession no. EU9082205). *varO* exon I codes for the extracellular region of the protein. The *varO* exon II sequence encoding the acidic terminal segment (ATS) is a partial sequence. The amino acid sequence boundaries of each domain, defined using previously described criteria (78, 80), are indicated.

genome or previously described sequence. Thus, no *varO* sensu stricto ortholog was found in any of the genomes investigated, reflecting the internal mosaic structure of the *var* genes (28, 38, 43). A search for individual domain orthologs in the 3D7 *var* gene repertoire (28, 38) identified DBL1 α_1 and DBL2 β orthologous domains in the PF13_0003 *var* gene, which, however, has a markedly different CIDR γ domain (30.3% sequence identity) (Table 2). The 3D7 orthologs of *varO* DBL4 β and *varO* DBL5 β were from the PF08_0141 *var* gene. The *varO* CIDR γ sequence had limited homology with the 3D7 *var* gene repertoire, and the highest level of identity was 47.2% identity with the PFD0995c CIDR2 γ sequence. The *varO* exon II se-

quence was a partial sequence, but it had specific signatures. The best match for this sequence in the 3D7 genome was PF11_0521. Interestingly, the 3D7 orthologs of *varO* DBL1 α_1 , DBL2 β , DBL4 β , and DBL5 β and the probable exon II ortholog are group A *var* genes (43).

Each *varO* domain had an ortholog with high sequence identity in the genomes of the IT4, HB3, Dd2, and Ghana isolates (32, 38, 87), but for each individual domain the identity scores were different for the different isolates (Table 2). Interestingly, each domain also had an ortholog in the *P. reichenowi* genome (32), and some of the identity scores were remarkably high (Table 2). The *varO* DBL1 α_1 domain had an ortholog with

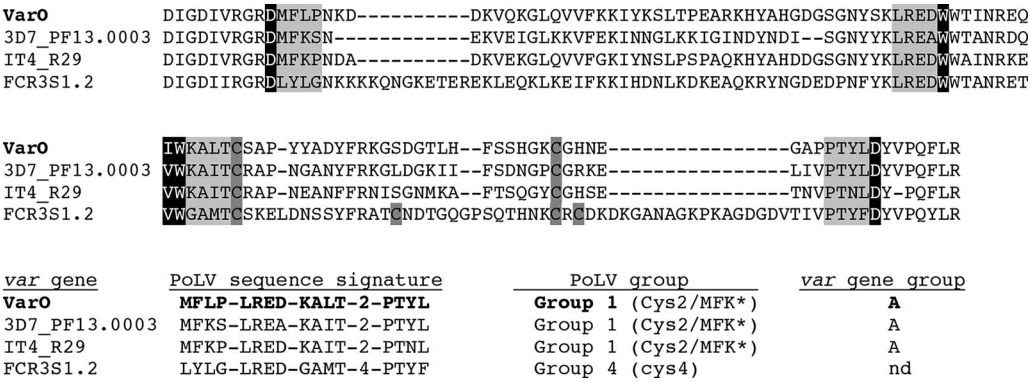


FIG. 2. PoLV classification of the varO DBL1 α_1 domain. The cysteine-PoLV classification approach was used to assign the varO DBL1 α_1 domain to a specific group (9, 10). The DBL1 α domains encoded by *var* genes in the IT4/FCR3 genome with documented involvement in rosetting, namely the varO nearest ortholog IT4/R29 (also designated *IT4var9*) (68) and FCR3S1.2 (also designated *IT4var21*) (14, 15) were included, as were the nearest orthologs of the varO DBL1 α_1 and IT4/R29 DBL1 α_1 domains in 3D7 (PF13_0003). The PoLV sequence features and the cysteine residues are indicated by a gray background. The conserved amino acids are indicated by a black background. The derived sequence signature and the assigned PoLV group for each DBL1 α domain are indicated in the lower panel. nd, not defined.

>60% identity in each *P. falciparum* genome investigated and thus seems to be the most conserved domain in the isolates. Interestingly, the highest sequence identity to varO DBL1 α_1 was found with the DBL1 α_1 domain of varR29 (67.1% identity) (annotated *IT4var9* in the IT4 *var* gene repertoire [38]), which is also implicated in rosetting (68). The DBL1 α domain of the FCR3S1.2 rosetting clone (14) (annotated *IT4var21* [38]) had a lower level of sequence identity (41.3%). The varO DBL5 β domain exhibited 81.4% sequence identity with the PF08_0141 DBL5 locus in the 3D7 genome, which is the highest level of identity found for the varO exon I domains. However, the nearest orthologs of varO DBL5 β in the other *P. falciparum* clones or isolates exhibited lower levels of sequence identity (Table 2). The least conserved domain was varO CIDR1 γ , for which the highest level of sequence identity was 51.8% (*IT4var8* CIDR1 γ). In the *P. reichenowi* genome, the levels of identity varied with the individual domains, and the highest level was observed for varO DBL4 β (68.5%).

The varO gene codes for a PfEMP1 adhesin mediating rosetting. To ascertain whether the dominant cDNA isolated from varO parasites did indeed encode the PfEMP1 adhesin mediating varO rosetting, individual domains were expressed on the surface of Cos7-L cells as described previously (37) and tested for binding to uninfected *Saimiri* RBC. Surface expression of DBL1 α_1 was associated with binding to uninfected RBC from *Saimiri* monkeys (data not shown), but the expression levels and the numbers of transfected cells were very low (1 to 3%). However, transfection of Cos7-L cells with a recodoned version of the DBL1 α_1 domain (rhDBL1 α) improved the surface expression and subsequent binding to human RBC (72).

To further analyze the binding capacity of varO domains, three other domains were recodoned. varO rhDBL1 α , rCIDR γ , rDBL2 β C2, and rDBL5 β were then cloned in baculovirus and expressed in High-5 insect cells. We chose the baculovirus-insect cell expression system because it has proved to be effective for the production of correctly folded cysteine-rich domains, including DBL domains (5, 17, 33, 63, 73). Surface exposure was monitored by S-IFA using an anti-His tag MAb,

and the level of cells expressing each construct was determined by flow cytometry. For each domain, the expression level and the number of insect cells expressing the recombinant protein were very high (Fig. 3A). Binding to O⁺ and A⁺ human RBC was readily seen on the surface of varO rhDBL1 α -expressing cells; the right panels of Fig. 3B1 show the results of two representative experiments with O⁺ RBC. There was no binding by surface-expressed recombinant varO rCIDR γ , rDBL2 β C2, or rDBL5 β , and Fig. 3B2 shows representative examples. The involvement of only DBL1 α_1 in erythrocyte binding was further confirmed by analysis of binding inhibition using varO DBL1 α_1 -specific antisera (see below).

In vitro varO model of rosetting and autoagglutination. The 89F5 varO clone was established in continuous in vitro culture in human O⁺ RBC from an infected *Saimiri* monkey (see Materials and Methods). The human RBC infected with 89F5 varO parasites had knobs and retained the surface cytoadherence phenotype (formation of rosettes and autoagglutinates) (Fig. 4A). Unlike rosettes, which were observed even at low levels of parasitemia, autoagglutinates were observed only in cultures with $\geq 8\%$ parasitemia. These two cytoadherence phenotypes were not platelet dependent since all cultures were grown with platelet-poor sera. This suggests that rosette formation and autoagglutinate formation are two distinct manifestations of the same cytoadherence phenotype that depend on the abundance of the respective binding partners. In this regard, varO autoagglutinates may be similar to the “giant rosettes” described by Heddini et al. (30). In addition, in line with previous findings with other rosetting clones and lines (14, 21, 49, 68), varO rosettes and autoagglutinates in human RBC were human serum dependent, suggesting that serum factors may be involved in these cytoadherence phenotypes.

Importantly, as observed with *Saimiri* RBC, in human RBC the 89F5 varO parasites expressed the varO gene. RT-PCR across the intron (encompassing DBL5 β -exon II or ID3-exon II) generated a band that was the size predicted for a correctly spliced product (Fig. 4B). The fragment observed was derived from RNA, since it was not observed when RT was omitted. The varO surface serotype, as defined with sera collected from

TABLE 2. varO domain orthologs and protein sequence identity in *P. falciparum* and *P. reichenowi* isolates^a

Parasite	DBL1 α 1		CIDR γ		DBL2B		DBL3 γ		DBL4B		DBL5B	
	var gene or contig	Identity %	var gene or contig	Identity %	var gene or contig	Identity %	var gene or contig	Identity %	var gene or contig	Identity %	var gene or contig	Identity %
3D7	PF13_0003 ^b	65.4	PFD0995c	47.2	PF13_0003 ^b	56.2	PFF0010w	54.5	PF08_0141 ^b	66.4	PF08_0141 ^b	81.4
IT4	IT4var9/R29var ^b	67.1	IT4var8 ^b	51.8	IT4var2 ^b	56.3	IT4var6 ^b	47.3	IT4var13	48.6	IT4var8 ^b	41.6
HB3	HB3var5 ^b	66.7	HB3var23	48.4	HB3var16	54.7	HB3var48 ^c	55.1	HB3var8	67.6	HB3var8	50.6
Dd2	Contig_1.57	67.3	Contig_1.924	45.8	Contig_1.227	55.4	Contig_1.9	54.5	Contig_1.181	50.3	Contig_1.24	37.8
Ghana	Contig_20793	64.8	Contig_18537	50.9	Contig_20179	58.8	Contig_21033	45.9	Contig_21299	68.9	Contig_20860	50.4
<i>P. reichenowi</i>	Contig_000023499	55.3	Contig_000023127	40.3	Contig_000023807	54.9	Contig_000023387	54.1	Contig_000023779	68.5	Contig_000022699	64.7

^a For each domain, sequence homology searches were performed using the genome sequences of the *P. falciparum* 3D7, IT4/25/5, HB3, Dd2, and Ghana isolates and *P. reichenowi*, the chimpanzee malaria parasite. The values are the levels of sequence identity. For each varO domain, the var gene with the highest sequence identity is indicated by bold type.

^b Group A/LpsA var gene.

^c Pseudogene.

89F5 varO-infected *S. sciureus*, was displayed by the parasites cultured using human RBC. Surface reactivity, as analyzed by S-IFA and flow cytometry (Fig. 4C) and by epifluorescence surface microscopy (Fig. 4D), clearly showed that human-adapted varO parasites were specifically recognized by sera from *Saimiri* monkeys infected with varO parasites but not by sera from animals infected with the sibling varR variant. Like 89F5 varO-infected *Saimiri* RBC (47), 89F5 varO-infected human RBC did not bind nonimmune *Saimiri* IgG or IgM (Fig. 4C). Previous work showed that uncloned varO-infected *Saimiri* RBC reacted with hyperimmune human sera (24). The 89F5 varO-infected human RBC exhibited this surface reactivity with a pool of hyperimmune human sera from Senegal (Fig. 4C and 4D) and did not react with nonimmune human sera. Here the surface reactivity was also due to IgG and not IgM.

Antisera raised to the rNTS-DBL1 α 1 domain react with the surface of 89F5 varO-iRBC and disrupt rosettes. To confirm that the varO gene was responsible for the surface phenotype and serotype assigned to varO parasites, we produced a soluble varO DBL1 α 1 recombinant protein to raise specific antisera. The rhDBL1 α 1 expression product used in the High-5 surface-binding assay described above proved to be insoluble. Accordingly, we redesigned the construct to contain the segment from Met-1 to Ser-487 (which includes the 95-residue N-terminal NTS region); thus, it contained 20 cysteine residues instead of the 14 cysteine residues in the first construct (Fig. 1). The new construct had much improved solubility and expression levels. The recoded varO rNTS-DBL1 α 1 gene sequence was optimized for the codon usage of the baculovirus-insect cell expression system (see Materials and Methods). Using this new construct, we obtained a 58-kDa soluble, secreted recombinant protein, varO rNTS-DBL1 α 1, that was purified to homogeneity as judged by SDS-PAGE analysis under reducing conditions (Fig. 5A). N-terminal sequencing indicated that there was a single N terminus corresponding to cleavage at the predicted site after the mellitin signal sequence (data not shown). The protein reacted on an immunoblot with an anti-His tag MAb (data not shown). Thus, the full-length recombinant protein was expressed.

The varO rNTS-DBL1 α 1 protein exhibited the predicted specificity pattern as determined by ELISA; it was recognized by sera from varO-infected monkeys but not by sera from varR-infected monkeys (Fig. 5B). Interestingly, it reacted with a pool of sera from Senegalese adults living in an area where malaria is endemic. Mice immunized with the soluble varO rNTS-DBL1 α 1 domain produced high antibody titers in ELISA (end point titer, 1:1,638,400) (Fig. 5C). Anti-varO rNTS-DBL1 α 1 antisera also reacted with the surface of live 89F5 varO-infected human RBC (Fig. 5D and insets), which they also agglutinated (data not shown). Importantly, these anti-varO rNTS-DBL1 α 1 mouse antisera disrupted the 89F5 varO rosettes, unlike preimmune sera or sera raised to irrelevant control proteins, such as DBL3 γ 732 (Fig. 5E). The end point titer for 89F5 varO surface-reactive antibodies was substantially higher (1:8,000) than that for rosette-disrupting antibodies (1:320).

Positive selection of varO parasites by panning. The var gene repertoire of Palo Alto is not known at present; our data indicate that it includes at least two rosetting types designated varO and varS (22, 24, 45), which display a different serotype.

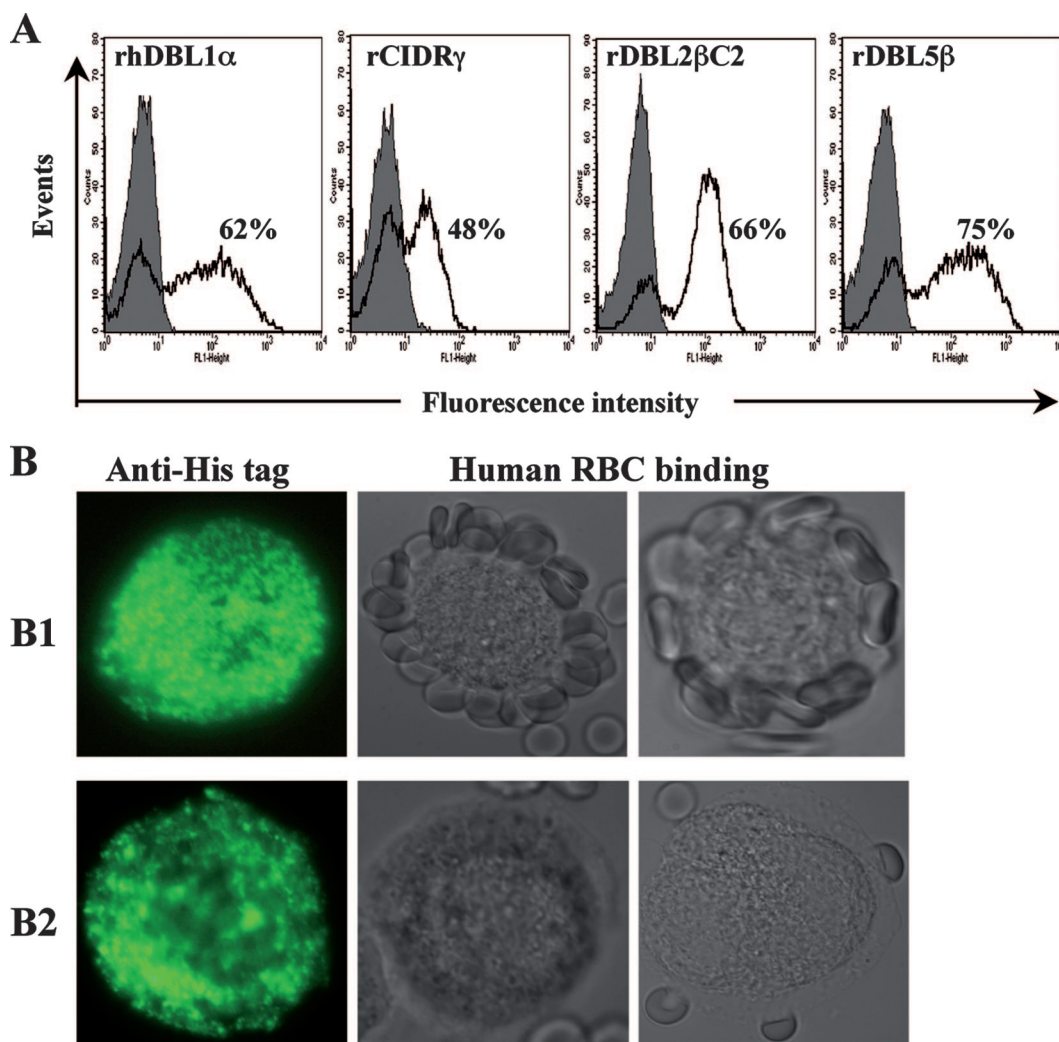


FIG. 3. Human RBC binding capacity of *varO* domains expressed on the surface of insect cells infected with recombinant baculovirus. The C-terminal His₆-tagged recoded *rhDBL1 α* , *rCIDR γ* , *rDBL2 β C2*, and *rDBL5 β* domains of *varO* were cloned into baculovirus, which was used to infect High-5 insect cells. The sequence boundaries of each construct are shown in Fig. 1. (A) Surface expression of the *varO* domains on High-5 infected cells was monitored by S-IFA using an anti-His tag MAb and a goat anti-mouse IgG Alexa Fluor 488-conjugated antibody. The level of expression of each construct was analyzed by flow cytometry. The shaded histograms show labeling in the absence of the anti-His tag MAb (incubation with only the conjugate). The percentage of High-5 cells expressing each construct is indicated. (B) Representative results for human RBC binding to the surface of High-5 cells expressing *rhDBL1 α* (panels B1) or *rCIDR γ* (panels B2). The absence of human RBC binding to the surface of High-5 cells expressing *rDBL2 β C2* and *rDBL5 β* is not shown. The left panels show surface expression of *varO* domain constructs by High-5 cells as determined by a fluorescence microscopy analysis of S-IFA generated using anti-His tag MAb. The right panels show the human RBC binding capacity of *varO* domains expressed on parallel cultures of High-5 infected cells. Representative results of two independent binding experiments performed with human O⁺ RBC are shown. Only the *varO* *rhDBL1 α* domain displayed RBC binding capacity.

However, based on the sequenced genome of IT4/FCR3 (38), we predict that four or five *var* genes are involved in rosetting in the Palo Alto genome. Weekly enrichment of rosettes by selective centrifugation did allow maintenance of rosette-forming parasites at a high rate but did not ensure long-term maintenance of a pure *varO* rosetting sero/phenotype in culture. Indeed, the preparation containing rosettes obtained after several months of continuous in vitro culture in human RBC contained additional rosette-forming variants that did not react with *varO* rNTS-DBL1 α ₁ mouse antisera (Fig. 6, upper panel). Moreover, unlike *varO*-iRBC, these non-*varO* rosetting variants were able to bind nonimmune human IgG and were recognized by hyperimmune human sera, which hampered assessment of the *varO*-specific surface reactivity. To

circumvent the progressive loss resulting from antigenic variation, *varO*-iRBC were positively selected by panning with MAb D15-50, a mouse MAb raised to *varO* rNTS-DBL1 α ₁ (see Materials and Methods). This panning procedure, which was carried out bimonthly, allowed a pure *varO* rosetting sero/phenotype to be maintained with >95% of the mature stages of the 89F5 *varO*-iRBC cultured in human RBC expressing the *varO* serotype (Fig. 6, lower panel), thus creating a stable in vitro model of *varO* rosetting. Such homogeneous parasite cultures expressing the PfEMP1*varO* adhesin were used to analyze seroprevalence in an African setting.

Very high seroprevalence for *varO* at the rural community level in a Senegalese setting where malaria is endemic. As shown above, 89F5 *varO* parasites infecting both *Saimiri* and

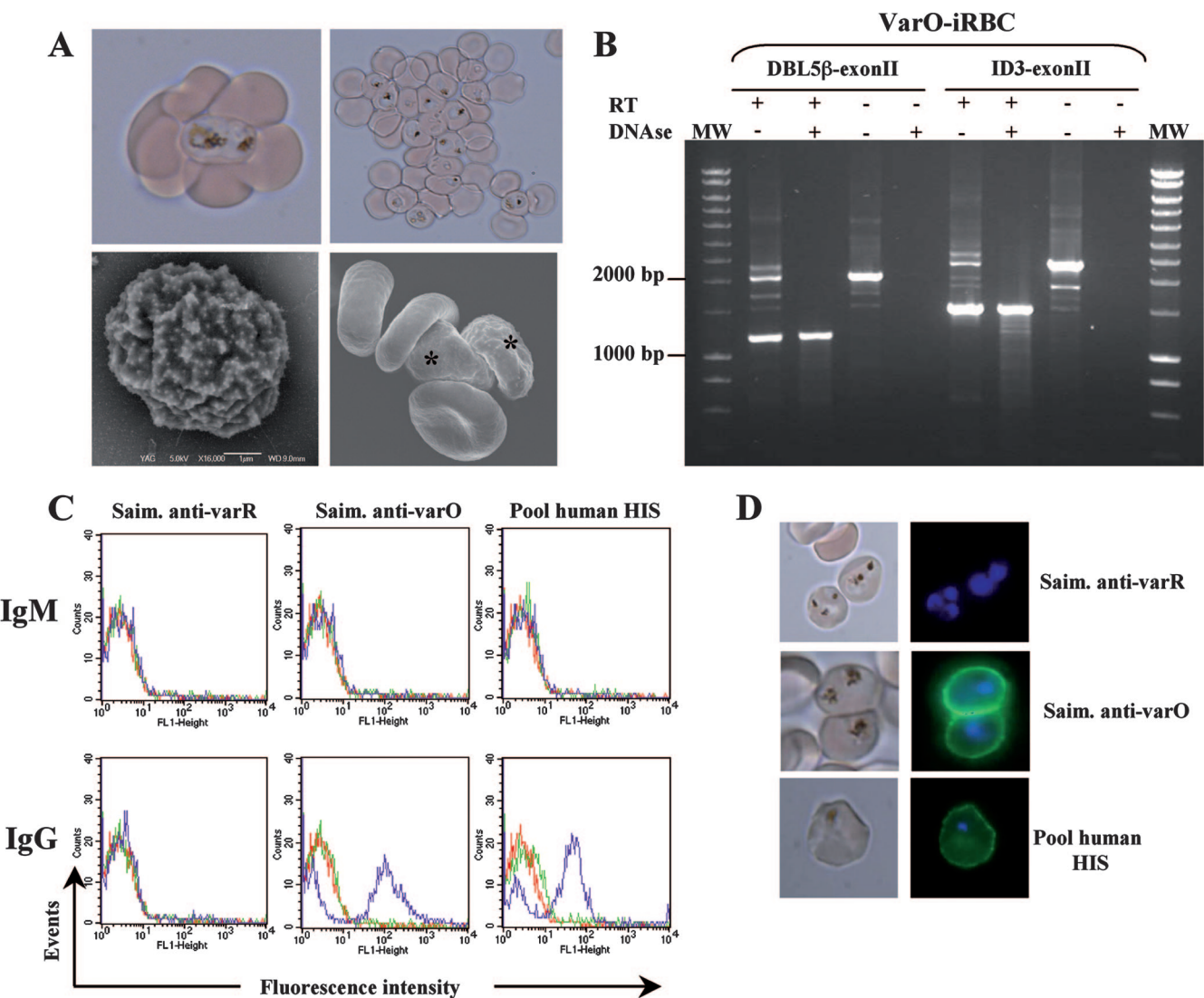


FIG. 4. Phenotype and serotype of the 89F5 varO rosettes cultivated in vitro in human RBC. (A) 89F5 varO parasites cultivated in human RBC at trophozoite and early-schizont stages are Knob⁺ parasites that are able to form rosettes and autoagglutinates. The upper panels show rosettes and autoagglutinates of varO-iRBC as viewed by light microscopy, and the lower panels show the results of an analysis of varO-iRBC by scanning electron microscopy. Scale bar = 1 μm. The asterisk indicates varO-iRBC. (B) *varO* gene expression in 89F5 varO cultures: RT-PCR amplification patterns for *varO* mRNA extracted from mature stages of rosetting varO parasites using the 5DBL5Bfo-VarO3exon2Brev primer pair (predicted size of genomic DNA, 2,050 bp; predicted size of mRNA, 1,158 bp) or the 5ID3Bfo-VarO3exon2Brev primer pair (predicted size of genomic DNA, 2,400 bp; predicted size of mRNA, 1,479 bp). Control reactions were carried out using samples treated with DNase or without reverse RT as indicated above the lanes. PCR products were analyzed by agarose gel electrophoresis. Representative results of three independent experiments are shown. Lanes MW contained molecular size markers. (C and D) S-IFA analysis of IgM or IgG binding to the surface of human RBC infected by mature-stage 89F5 varO parasites. Binding of nonimmune (green lines) or immune (purple lines) *Saimiri* (Saim.) or human antibodies reacting with varO surface-exposed antigens was monitored using a goat anti-human IgM or IgG Alexa Fluor 488-conjugated antibody. A varO-iRBC preparation incubated with the conjugate alone was included in each experiment (red lines). varO-iRBC were stained with Hoechst dye (nuclei stained blue). S-IFA results were analyzed by flow cytometry (C) and fluorescence microscopy (D). The results are representative of at least three independent experiments. HIS, hyperimmune sera.

human RBC were surface positive when they were tested with a pool of hyperimmune sera from Dielmo volunteers. When sera were tested separately, each individual serum from this pool reacted with 89F5 varO parasites, ruling out the possibility of a pool artifact. To investigate the seroprevalence for the culture-adapted 89F5 varO parasites at the village level (i.e., the seroprevalence in volunteers in all age groups living in a defined setting where malaria is endemic), we studied IgG

surface reactivity by flow cytometry using an archived collection of sera collected from 235 of the 247 villagers living in Dielmo. A very high proportion (93.6%; 95% confidence interval [CI], 89.7 to 96.4%) of Dielmo villagers had antibodies that reacted with the surface of 89F5 varO-iRBC (Fig. 7A, left panel). There was a marked age dependence (Fig. 7B, left panel), with low seroprevalence in the <2-year-old children (prevalence, 33%; median surface reactivity, 16.48 AU; 25 to

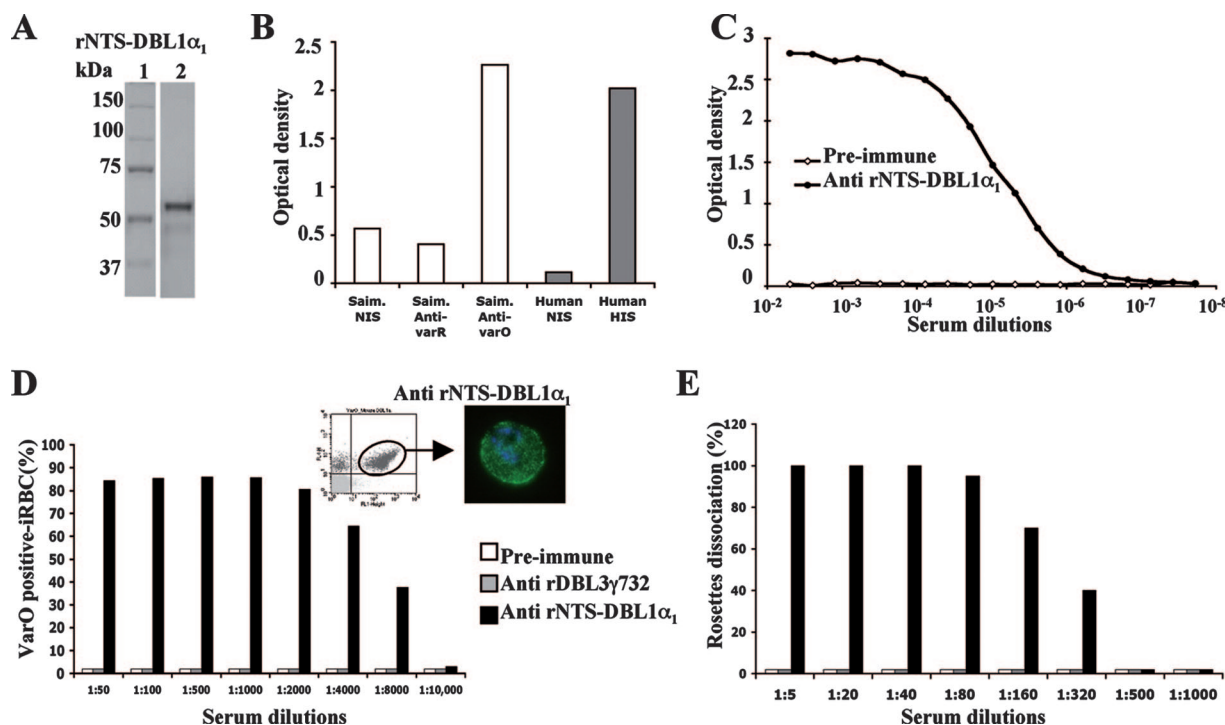


FIG. 5. varO rNTSDBL1 α_1 recombinant protein elicits surface-reacting and rosette-disrupting antibodies. (A) The soluble rNTS-DBL1 α_1 recombinant protein (3.5 μ g, 58 kDa) was loaded on a 10% SDS-PAGE gel under reducing conditions and stained with Coomassie blue. Lane 1, molecular mass standards; lane 2, rNTS-DBL1 α_1 recombinant protein. (B) The reactivity of *Saimiri* sera (open bars), including *Saimiri* nonimmune sera (Saim. NIS) and immune sera (Saim. Anti-varO and Saim. Anti-varR), and human sera (filled bars), including pools of human hyperimmune sera from adults living in Dielmo (Human HIS) or non-malaria-immune European blood donors (Human NIS), was tested by using ELISA and the soluble varO rNTS-DBL1 α_1 domain. The optical densities are indicated. The results are representative of three independent experiments. (C to E) Soluble rNTS-DBL1 α_1 and rDBL3 γ 732 domains were used to immunize groups of five outbred (OF1) mice. The pools of preimmune, anti-rNTS-DBL1 α_1 , and/or anti-rDBL3 γ 732 mouse sera were then analyzed using ELISA (C), S-IFA and flow cytometry (D), and the rosette dissociation assay (E). (C) Titration of anti-rNTS-DBL1 α_1 sera on the rNTS-DBL1 α_1 recombinant protein by ELISA. Twofold serial dilutions were prepared, ranging from 5×10^{-3} (1:200 dilution) to 2×10^{-8} (1:52,428,800 dilution). The pool of preimmune sera, which was used as a control, was negative. (D) Representative results obtained for titration of preimmune, anti-rDBL3 γ 732, and anti-rNTS-DBL1 α_1 antibodies with live varO-iRBC by S-IFA and flow cytometry. For each dilution tested, the percentage of surface-positive varO-iRBC was calculated. The left inset shows a representative histogram obtained by flow cytometry (FL1, anti-human IgG Alexa Fluor 488 staining; FL4, Hoechst dye staining of varO parasite culture); varO NTS-DBL1 α_1 -positive cells are indicated. The right inset shows S-IFA staining of varO-iRBC visualized by epifluorescence microscopy. (E) Rosette dissociation assay results obtained after incubation of varO rosettes (>90% rosette-forming parasites) with serial dilutions of preimmune, anti-rNTS-DBL1 α_1 , and anti-rDBL3 γ 732 mouse sera. The percentage of rosetting parasites in the presence of preimmune sera was used as the reference. The rosette dissociation capacity of each antiserum pool is indicated. Each assay was performed in duplicate.

75% interquartile range [IQ25-75], 8.15 to 21.05 AU), higher prevalence in the 2- to <5-year-old age group (prevalence, 78%; median surface reactivity, 79.23 AU; IQ25-75, 24.65 to 93.43 AU), and very high prevalence in the >5-year-old group (prevalence, 98%; median surface reactivity, 96.2 AU; IQ25-75, 90.05 to 101.08 AU). The reactivity index (which takes into account the MFI and the percentage of varO-positive iRBC) showed similar age-associated acquisition of varO surface-reactive antibodies.

We next assessed the seroprevalence of the same sera with the varO rNTS-DBL1 α_1 recombinant protein by performing ELISA. The percentage of antibodies reacting with the rosetting domain of the PfEMP1varO adhesin was very high (87.7%; 95% CI, 82.8 to 91.6%), and it increased with age, from a low value (prevalence, 11.1%; median, 2.38 AU; IQ25-75, 0.89 to 3.42 AU) in the <2-year-old children to an elevated value (prevalence, 65%; median, 35.65 AU; IQ25-75, 3.76 to 123.57 AU) in the 2- to <5-year-old children to a very high value (prevalence, 94%; median, 71.18 AU; IQ25-75, 42.39 to

104.5 AU) in the >5-year-old group (Fig. 7A and 7B, right panels). The varO surface-reactive antibodies were positively correlated with the ELISA reactivity to varO rNTS-DBL1 α_1 (Fig. 7C), as determined by Spearman's rank correlation test (for surface reactivity versus rNTS-DBL1 α_1 , $\rho = 0.60$ [$P < 0.0001$]; for reactivity index versus rNTS-DBL1 α_1 , $\rho = 0.57$ [$P < 0.0001$]).

DISCUSSION

Our findings show that 89F5 varO parasites adapted to culture in human RBC express the same *var* gene, the same rosetting and autoagglutination cytoadherence phenotype, and the same surface serotype as the original uncloned varO line and the 89F5 varO clone propagated in *S. sciureus* monkeys. Mature varO parasites expressed a *var* gene that we designated varO, which possesses group A *var* gene characteristics and a varO DBL1 α_1 PoLV group 1 (Cys2) sequence signature, two molecular features associated with rosetting and/or severe ma-

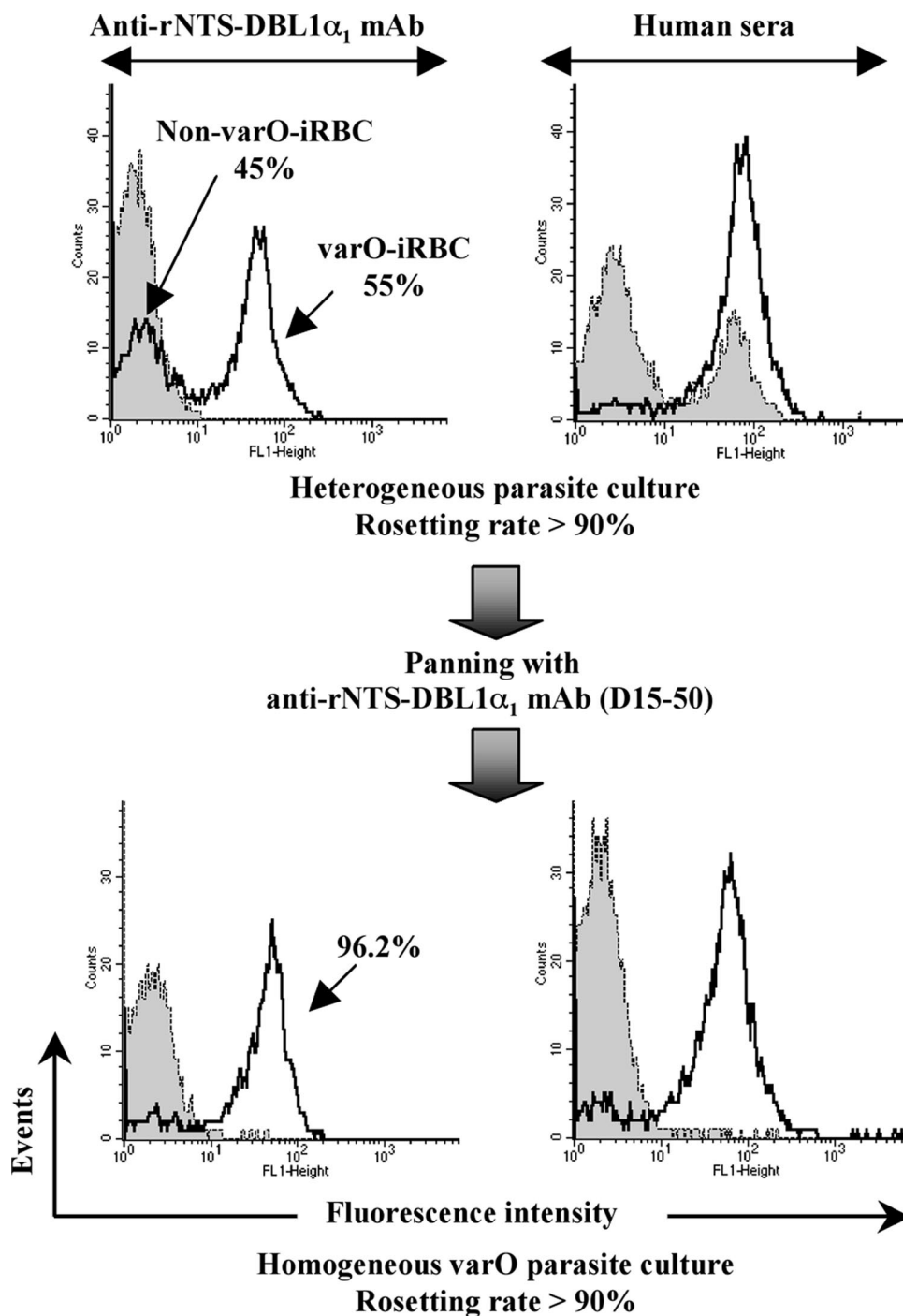


FIG. 6. Specific selection of varO-iRBC by panning with an anti-rNTS-DBL1 α_1 MAb. varO parasites were maintained in continuous *in vitro* culture in human RBC. The surface reactivities obtained after several weeks of culture and several rounds of rosette enrichment by centrifugation on ice-cold Ficoll (upper panels) and after positive selection of iRBC expressing the varO NTS-DBL1 α_1 domain by panning with MAb D15-50 directed against the varO rNTS-DBL1 α_1 protein (lower panels) are shown. Mature-stage parasites were stained with a pool of preimmune mouse sera and with MAb D15-50 (left panels) or with pools of nonimmune or hyperimmune (Dielmo) human sera (right panels). The surface reactivity on iRBC was monitored by S-IFA and flow cytometry using a goat anti-mouse or anti-human IgG Alexa Fluor 488-conjugated antibody. The shaded histograms show labeling with nonimmune sera. The S-IFA reactivity obtained with immune sera is indicated by open histograms. As indicated, after panning of a heterogeneous culture, >95% of mature parasites expressed the varO NTS-DBL1 α_1 domain and had a typical varO serotype (i.e., they did not bind nonimmune human IgG).

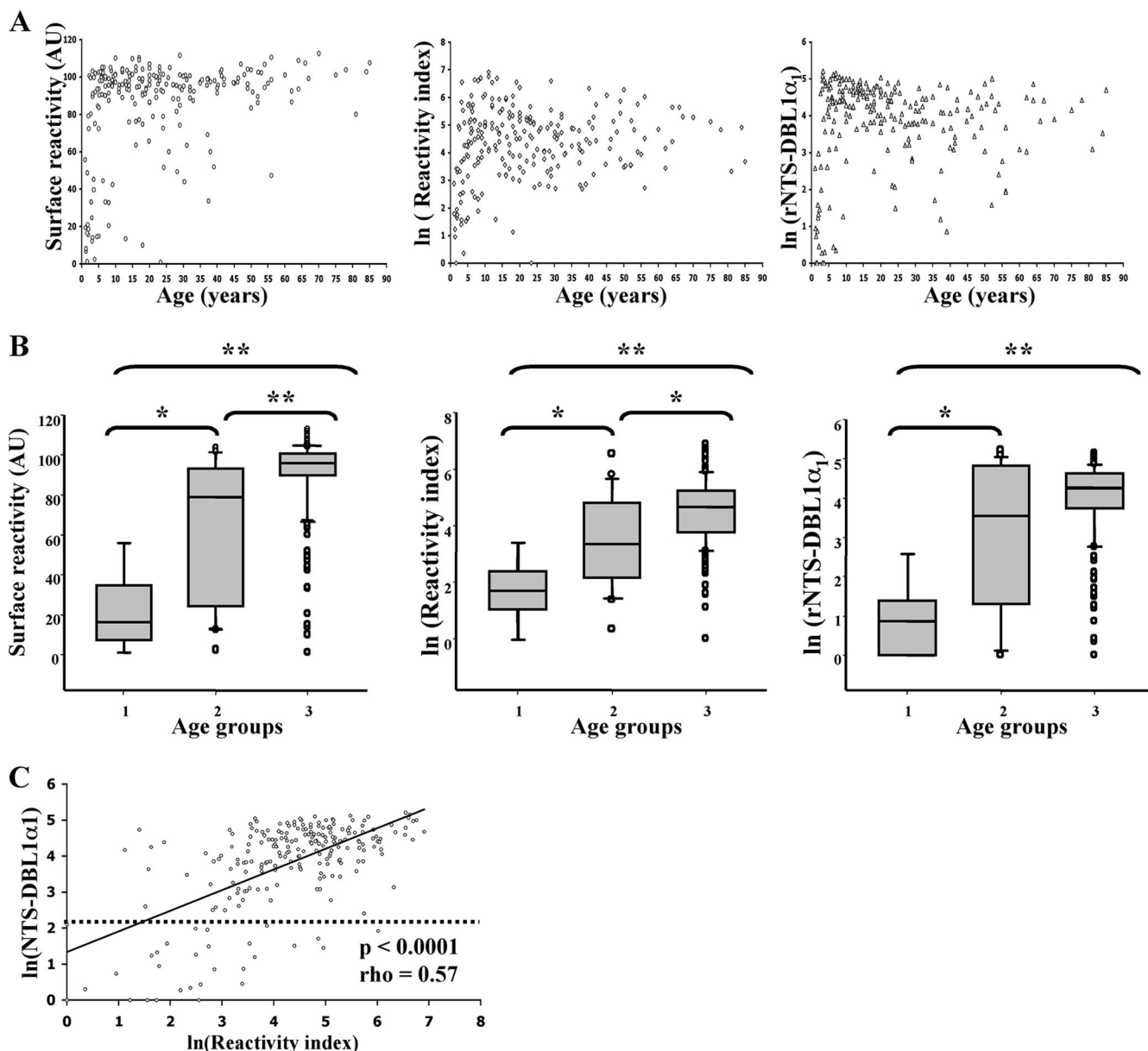


FIG. 7. Seroprevalence for 89F5 varO-iRBC and varO rNTSDBL1 α_1 recombinant domains at the village level in Dielmo (Senegal). Blood samples were obtained from 235 (ages, 1 to 85 years; mean age, 23 years) of 247 villagers living in Dielmo during the 1992 transmission season. For each villager, antibodies (IgG) reacting with the surface of live varO-iRBC were analyzed by S-IFA and flow cytometry. Antibodies against the varO rNTS-DBL1 α_1 domain were analyzed by ELISA. The results were expressed in AU (see Materials and Methods) and log transformed (ln) to obtain an approximately normal distribution. (A) Age distribution of varO-iRBC surface-reactive antibodies (expressed as surface reactivity and reactivity index) and rNTS-DBL1 α_1 -reactive antibodies. (B) Acquisition of surface- and rNTS-DBL1 α_1 -reactive antibodies in three age groups, group 1 (<2 years old; $n = 9$), group 2 (2 to <5 years old; $n = 23$), and group 3 (>5 years old; $n = 203$). The boundaries of the boxes indicate the 25th and 75th percentiles, and the line in each box indicates the median. The whiskers above and below the boxes indicate the 90th and 10th percentiles. The outlying dots indicate values exceeding the 90th and 10th percentiles. The results of Wilcoxon's nonparametric test are indicated by asterisks, as follows: *, $P < 0.01$; **, $P < 0.0001$. (C) Linear regression of ln(rNTS-DBL1 α_1) (ordinate) as a function of ln(reactivity index) (abscissa). The estimated regression equation is $f(x) = 1.3947 + 0.5550x$, and the correlation coefficient (r^2) is 0.34. The Spearman rank correlation coefficient (ρ) and the P value are indicated.

laria in African children (9, 41, 59). Establishment of an in vitro model with a homogeneous parasite population expressing the *varO* gene and the varO sero/phenotype (i.e., a "single-variant" culture of the 89F5 clone) allowed us to analyze with a high degree of confidence varO field seroprevalence. The results obtained showed that the varO rosetting and autoag-

glutination serotype was very prevalent in Dielmo, a Senegalese setting where malaria is holoendemic, and moreover that varO seroconversion occurs early in life. It is interesting to place these characteristics in the perspective of the acquired immunity to severe malaria of young African children, which correlates with seroconversion to so-called frequent PfEMP1 types

(11, 57) associated with group A genes (33, 34). The age profile for seroconversion for varO parasites in Dielmo and the varO cytoadherence characteristics are consistent with varO classification in the subset of group A genes implicated in malaria pathogenesis.

As found with other var genes expressed by rosette-forming parasites (14, 68), the varO DBL1 α_1 domain mediated erythrocyte binding on the surface of Cos7-L cells. This was observed both when the wild-type sequence was used and when a recodoned sequence devoid of N-glycosylation sites was used. The same DBL1 α_1 coding sequence also mediated binding to human RBC when it was expressed on the surface of insect cells. Furthermore, antibodies raised to the soluble recombinant varO NTS-DBL1 α_1 domain reacted with high titers to the surface of 89F5 varO-iRBC and totally disrupted varO rosettes. This is convincing evidence that the varO rosetting is indeed mediated by the varO NTS-DBL1 α_1 domain encoded by the varO gene. Using in vitro cultures with >90% of the rosetting parasites stained positively with the anti-NTS-DBL1 α_1 antibodies (and hence expressing varO), both rosetting or autoagglutinates and giant rosettes were observed, suggesting strongly that PfEMP1varO mediates both rosetting and autoagglutination. It is worth noting that antibodies to varO NTS-DBL1 α_1 disrupted rosettes but did not dissociate autoagglutinates. Indeed, while serial dilutions showed a marked reduction in the number of rosettes, the number of agglutinates in the sample remained unchanged (except at dilutions inducing antibody-dependent iRBC agglutination, where all iRBC were observed in aggregates). Further work is needed to clarify this point and to identify the domain mediating platelet-independent varO autoagglutination.

Production of recombinant DBL domains with native folding is difficult, possibly because the richness of disulfide bridges constrains the domain structures. We have used the baculovirus-insect cell production system because, for example, it has been used successfully to produce cysteine-rich MSP1-19 EGF domains (18, 63) and has also been used for DBL domains (4, 5). A surface-expressed domain displaying binding activity was produced by using the domain boundaries originally defined by Smith et al. (77). However, this protein was insoluble and not secreted (despite being cloned in the vector used to secrete MSP1-19 [18]). Difficulty generating soluble DBL1 α domains is not new and, indeed, was confirmed by computer prediction by Ahuja et al. (1). Nevertheless, a soluble protein could be produced using a redesigned construct with an elongated domain, based on folding of the EBA-175 DBL domains (76). The C-terminal boundaries of PfEMP1 DBL domains are more difficult to identify because of substantial divergence from EBA in this region. However, we obtained an apparently properly folded protein using this approach, because the protein elicited high titers of surface-reacting antibodies and of rosette-disrupting antibodies. It is worth mentioning that mutation of the six N-glycosylation sites did not result in major alterations in the antigenicity. Another important consequence of using a recodoned version was the sequence stability of the plasmid constructs. The native uncoded sequence frequently mutated upon propagation in *Escherichia coli* and recloning in an expression vector. This was not observed once a recodoned version with a less biased G+C content was used.

The varO soluble rNTS-DBL1 α_1 domain elicited high anti-

body titers in mice that appeared to be better than those previously reported by other groups for other recombinant DBL domains. In particular, we observed surface reactivity at dilutions that were 10 to 100 times higher than the dilutions at which surface reactivity was observed for recombinant DBL1 α domains from other rosette-forming parasites using different expression systems, such as *E. coli* or Semliki Forest virus (16, 51). An important observation that we made with varO anti-rNTS-DBL1 α_1 antibodies raised in mice was that the rosette-disrupting titers were substantially lower than the titers for surface-reactive antibodies and rNTS-DBL1 α_1 ELISA-reactive antibodies. This suggests that only a fraction of surface-reactive antibodies react with the receptor-binding site and/or that only the antibodies with a very high affinity are able to disrupt rosettes. Nonetheless, the varO rNTS-DBL1 α_1 domain elicited titers of surface-reactive, rNTS-DBL1 α_1 -reactive, and/or cytoadherence-disrupting antibodies that were substantially higher than the titers obtained so far using other antigen production systems (5, 6, 16, 34). We attribute this to the baculovirus-insect cell expression and/or to the fact that the elongated construct enabled production of a recombinant domain with adequate conformational fidelity to the native protein and epitope presentation.

The in vitro varO model with cultivated human RBC is experimentally easier to handle than the in vivo infection model with *Saimiri* monkeys. To develop a model with all iRBC displaying the same PfEMP1varO adhesin and the varO-iRBC serotype, weekly rosette purification was not sufficient. Positive selection with an anti-rNTS-DBL1 α_1 antibody was necessary to eliminate the non-varO rosetting sibling variants enriched along with varO by centrifugation on ice-cold Ficoll (48). The varO model is thus the first rosetting model to be developed in this way. We anticipate that such a procedure will be necessary for the other var genes mediating rosetting and, more generally, for every var gene mediating a cytoadherence phenotype shared by multiple members of the var repertoire, such as CD36 binding. Working with a homogeneous parasite population expressing a single var gene and a single serotype is critical for seroprevalence and cross-reactivity studies, as well as for biochemical host receptor interaction studies.

The 89F5 varO parasites were shown here to express an iRBC surface serotype and an NTS-DBL1 α_1 serotype that have very high seroprevalence in Dielmo, Senegal. Palo Alto parasites originated from Uganda and were collected in 1978. We do not know whether the varO gene or its individual modular domains circulate as such in the west African setting analyzed here more than 14 years after Palo Alto was collected or whether the observed serologic reactions reflect strict varO-specific reactions or cross-reactions with varO-related sequences. To answer this question, analysis of the local var gene repertoire is needed, as is analysis of surface cross-reactivity, which is a vast undertaking in view of the large field diversity of var repertoires. The question of cross-reactivity between closely related variants is still open; there is some evidence of cross-reactivity with animal sera raised to individual domains (51), and there have been reports of limited cross-reactivity in the field with human sera. Mixed agglutination assays have indicated, at best, marginal cross-reactivity (56). The conservative conclusion from our data is that the varO serotype (if not the varO genotype, including mosaic genes with reshuffled

individual domains) is indeed frequent in this setting where malaria is endemic.

Using the recombinant NTS-DBL1 α_1 domain, which appears to be correctly folded since it elicits surface-reacting and rosette-disrupting antibodies, we could investigate seroprevalence for the first *varO* domain. The seroprevalence was high and correlated with surface reactivity. The two reactivities, however, did not fully overlap, suggesting that antibodies to other *varO* domains and/or surface antigens might contribute to surface reactivity. Cloning and expression of additional *varO* domains are under way to explore this hypothesis. This seroepidemiological survey also showed that the prevalence of antibodies reacting with *varO* parasites and/or *varO* rNTS-DBL1 α_1 increased early in life and was very high from the age of 5 years onward (5 years is the age at which the risk of malaria episodes drops substantially in the village studied) (83). In this regard, the seroprevalence for the *varO*-iRBC surface is much higher than reported for the FCR3S1.2 rosetting clone or field isolates in semi-immune Kenyan children (51). Additional work is in progress (in particular, a longitudinal follow-up study of individual children) to dissect the kinetics of acquisition of antibodies to the *varO*-iRBC surface and to individual domains and to study the functional role of these antibodies and their possible association with protection.

Analysis of severe malaria in human patients has identified a broad range of rosette, autoagglutinate, or platelet clumping frequencies (12, 13, 23, 30, 64, 67, 69, 85, 88). Only a minority of isolates display high rosette frequencies. Consistent with this, multiple *var* genes are expressed by the circulating parasite pool (42) and by the sequestered population (52, 53). Such heterogeneity precludes analysis of the contributions of specific parasite features to malaria pathology. We show here that the *varO* phenotype is related to severe malaria in several respects, including the cytoadherence phenotype, the elevated multiplication rate contributing to high parasite densities, the expression of a *var* gene having the characteristics of the group A/UpsA *var* gene subset, and the expression of a serotype with very elevated prevalence in semi-immune children. The Palo Alto *varO* model is the first in vitro and in vivo model for such combined phenotypes and provides a unique tool for exploring the contribution of rosetting or autoagglutination cytoadherence to the pathogenesis of malaria.

ACKNOWLEDGMENTS

The personnel in charge of animal care at the Pasteur Institute of French Guiana are warmly thanked. We are grateful to C. El Euch for her help with sequencing of the *varO* gene. We express our gratitude to the villagers of Dielmo, the medical staff, and our collaborators at the Institut Pasteur de Dakar and Institut de Recherche et de Développement de Dakar (in particular, C. Rogier, J. F. Trape, C. Sokhna, A. Touré-Baldé, and J. Faye) for epidemiological data and sample collection. We thank F. Marchand for her help with MAb production, A. Namane and J. D'Alayer for mass spectrometry and protein sequence analysis, and A. Mallet and N. Cayet for their technical assistance with scanning electron microscopy analysis. We thank P. David, N. Mohandas, and T. Fandeur for helpful comments on the manuscript.

This study received financial support from the French National Research Agency (Agence Nationale de la Recherche grant MIME 021 01-02); A.J. was supported by this Agence Nationale de la Recherche grant. This work was part of the activities of the BioMalPar European Network of Excellence supported by European grant LSHP-

CT-2004-503578 from Priority 1 "Life Sciences, Genomics and Biotechnology for Health" in the 6th Framework Program.

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